Changes in splicing and neuromodulatory gene expression programs in sensory neurons with pheromone signaling and social experience

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Abstract

Social experience and pheromone signaling in ORNs affect pheromone responses and male courtship behaviors in *Drosophila*, however, the molecular mechanisms underlying this circuit-level neuromodulation remain less clear. Previous studies identified social experience and pheromone signaling-dependent modulation of chromatin around behavioral switch gene *fruitless*, which encodes a transcription factor necessary and sufficient for male behaviors. To identify the molecular mechanisms driving social experience-dependent neuromodulation, we performed RNA-seq from antennal samples of mutant fruit flies in pheromone receptors and *fruitless,* as well as grouped or isolated wild-type males. We found that loss of pheromone detection differentially alters the levels of *fruitless* exons suggesting changes in splicing patterns. In addition, many Fruitless target neuromodulatory genes, such as neurotransmitter receptors, ion channels, and ion transporters, are differentially regulated by social context and

pheromone signaling. Our results suggest that modulation of circuit activity and behaviors in response to social experience and pheromone signaling arise due to changes in transcriptional programs for neuromodulators downstream of behavioral switch gene function.

Introduction

Detection of the social environment through pheromone signaling is critical for animals to recalibrate sex-specific behaviors such as mating and aggression (Curley et al., 2011; Cushing and Kramer, 2005; Dey et al., 2015; Sethi et al., 2019). It is thought that changes in social environment can modify the regulation of genes necessary for neuronal homeostasis, physiology, and transmission, ultimately affecting circuit function and behaviors (Cushing and Kramer, 2005; Flavell and Greenberg, 2008; West and Greenberg, 2011). Previous studies on the effects of early life experience have identified changes in neuroanatomy, synaptic plasticity, neurotransmission, and gene expression. For example, maternal licking and grooming of pups, increases DNA methylation around glucocorticoid receptor gene, leading to long-lasting effects on offspring stress responses and behaviors (McGowan et al., 2009; Mifsud et al., 2011; Weaver et al., 2004). However, transcriptional cascades driving in sensory and social experience-dependent modulation of gene expression, circuit function, and behaviors remain unclear.

Identifying gene regulation cascades by which social signals influence neural and behavioral responses requires a model system with well-defined circuits and genetic regulators with roles in neurophysiology, circuit structure, and behavioral function. Courtship circuit and behavior in *Drosophila melanogaster* is an excellent and unique model that provides an experimental system to address this question. In *Drosophila*, male-specific courtship behaviors are governed by a master transcriptional regulator Fruitless^{M} (Fru^{M}), which is encoded by male-specific alternative splicing of the *fruitless* (*fru*) gene from the P1 promoter (Dickson, 2008; Yamamoto

and Koganezawa, 2013). It is known that Fru^M is both necessary and sufficient for male courtship as loss of Fru^M in males leads to a loss of male-female-specific courtship interaction (Demir and Dickson, 2005; Ryner et al., 1996; Von Philipsborn et al., 2014). Fru^M is expressed in approximately 2000 interconnected neurons throughout the peripheral and central nervous system and its expression is required for the development, function, and plasticity of the circuit which drives male-specific behaviors (Yamamoto and Kohatsu, 2017). In particular, social cues such as pheromones can affect courtship behaviors in males (Dweck et al., 2015; Kurtovic et al., 2007; Lin et al., 2016; van der Goes van Naters and Carlson, 2007; Yamamoto et al., 2013; Yan et al., 2020). Two types of these pheromones, male-specific pheromone *cis*-vaccenyl acetate and non-sex-specific pheromones (such as methyl laurate and palmitoleic acid), activate Fru^M-positive olfactory receptor neurons (ORNs) expressing Or67d and Or47b receptors, respectively (Dweck et al., 2015; Kurtovic et al., 2007; Lin et al., 2016). These two ORN classes act differently, with Or67d regulating male-male repulsive behaviors and aggression, whereas Or47b functioning to drive male-courtship advantage with age and social experience (Dweck et al., 2015; Lin et al., 2016; Sethi et al., 2019; Wang et al., 2011). Previous studies have reported that different social contexts, as well as loss of Or47b or Or67d function, alter the regulation of *fru* transcription, particularly the enrichment of active chromatin marks around *fru* promoters (Hueston et al., 2016; Zhao et al., 2020). In addition, the expression of fru^M isoforms in Or47b and Or67d ORNs affects physiological responses to pheromone ligands and courtship behaviors (Lin et al., 2016; Ng et al., 2019; Sethi et al., 2019; Zhang and Su, 2020). It is likely that changes in social context, pheromone signaling, as well as subsequent changes in *fru* regulation, affect the expression of ion channels as well as neurotransmitter receptors with functions regulating neurophysiology. Indeed, Fru^M binding is detected upstream of many ion channels and genes controlling neural development and function in the central brain (Nojima et al., 2014; Vernes, 2014). Even though these studies point to the regulation of neuronal and circuit function by Fru^M, very little is known about how it

affects the expression of these target genes, or how pheromone signaling and social experience affect transcriptional programs by modulating Fru^M.

Here we performed antennal RNA-seq to determine transcriptional changes in response to social isolation and disrupted pheromone receptor signaling or Fru^M function. Our results show that *fru* splicing patterns are also modified in pheromone receptor mutants. We also found that transcriptional programs associated with neuromodulation are altered. Many of the Fru^M target neuromodulatory genes are misregulated in the same direction in fru^M and pheromone receptor mutants. These results are uncover a gene regulatory cascade from pheromone receptors to *fru* regulation, which alter neuromodulatory transcriptional programs to ultimately modulate neuronal and circuit responses in different social contexts.

Results

Neuronal transcriptional programs are modulated with social isolation and lack of pheromone receptors or Fru^M function.

To identify genes regulated in the peripheral olfactory system by social experience, pheromone signaling, and Fru^M, we utilized RNA-seq from whole antennae of 7-day old wild-type (w^{1118}) males that are either group-housed (w^{1118} *GH*) or single-housed (w^{1118} *SH*), as well as group-housed *Or47b* mutant males ($Or47b^1$), *Or67d* mutant males ($Or67d^{GAL4}$), and fru^M mutant males (fru^{LexA}/fru^{4-40}) (Figure 1A). Each condition included 3 biological replicates except for fru^{LexA}/fru^{4-40} with only two (Figure 1B). Each sample had mapped reads ranging between 24 and 40 million and hierarchical clustering analysis based on Pearson's correlation between samples shows consistency among replicates within the same genotype (Figure 1B). Principal component analysis (PCA) also shows the expected grouping of the replicates belonging to the same condition, across the first two principal components (PC) accounting for most of the overall variance (32% and 19%) (Figure 1C). We also found that gene expression changes were more

similar among *Or67d, Or47b,* and *fru*^{M} mutants, compared to grouped or isolated wild-type male antennae (Figure 1B,C). As expected, expression levels of *Or47b, Or67d*, and male-specific *fru* exon are significantly lower in all replicates for *Or47b*, Or67d, and *fru*^{M} mutants, respectively, though the changes of the whole *fru* gene locus cannot be detected (Figure 1D; Figure 4B), validating genotype-specific changes in each condition. In addition, genes known to be absent in adult antennae, such as *amos* (Goulding et al., 2000; Li et al., 2016; zur Lage et al., 2003), also show nearly no expression, whereas housekeeping genes, like *Act5C*, *Gapdh2*, *RpII18*, *fl(2)d*, and *wkd*, show nearly identical expression across all samples (Figure 1D). These results point to high RNA-seq data quality across sample groups and within biological replicates.

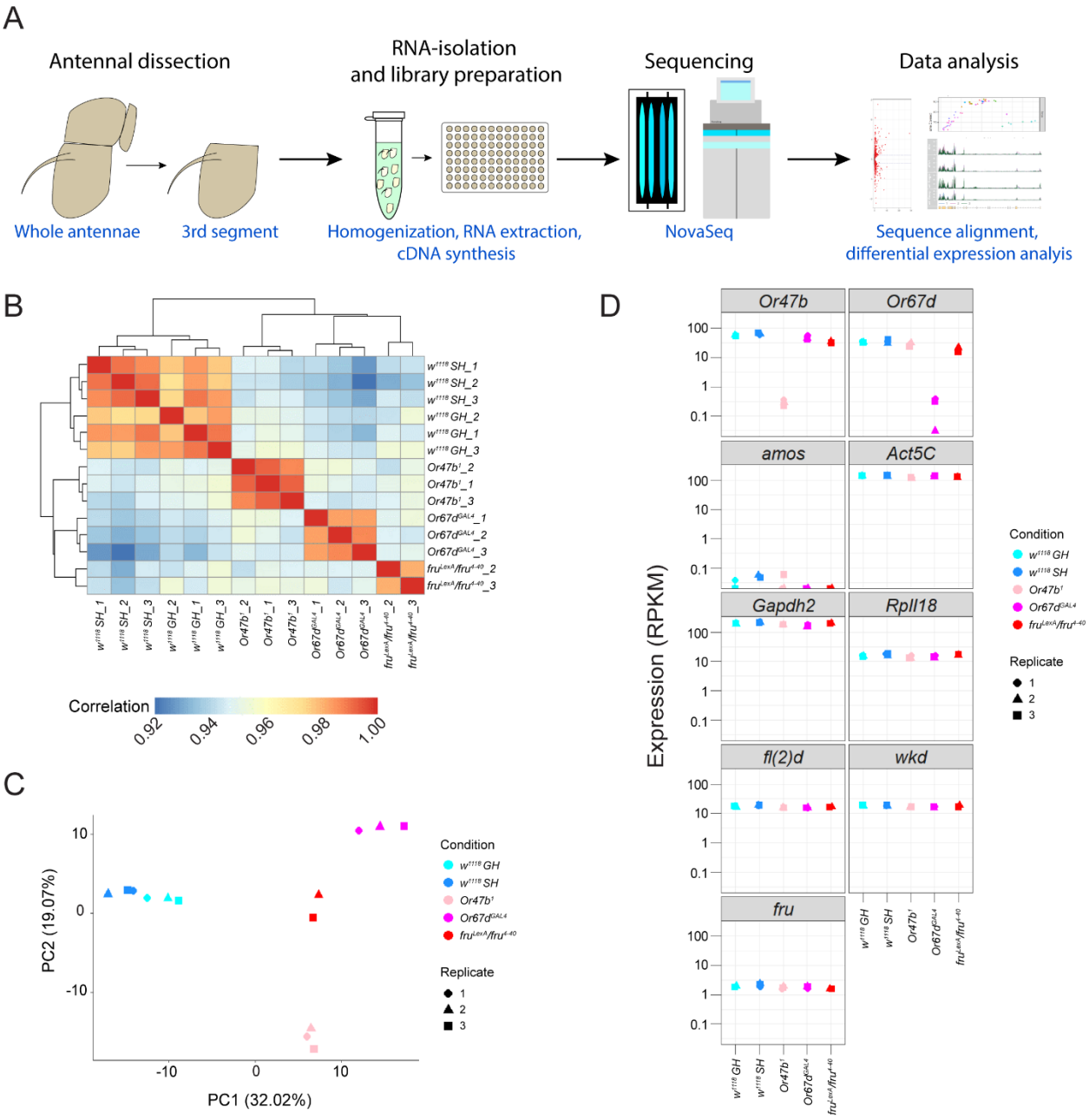

Figure 1. Overview of RNA-seq samples from male antennae. (A) schematic for antennal RNA-seq workflow. **(B-C)** Hierarchical clustering based on Pearson's correlation matrix **(B)** and PCA analysis **(C)** of transcriptional profiles among biological replicates from antennae of wild-type group-housed (*w*^{1118} *GH*), single housed (*w*^{1118} *SH*), and group-housed *Or47b*^{1}, *Or67d*^{GAL4}, and *fru*^{LexA}/*fru*$^{4-40}$ mutant male flies. **(D)** Transcript levels for several representative negative and positive control genes among all samples.

We then ran the differential expression analysis to globally examine the transcriptional changes upon loss of social expression, pheromone sensing, or Fru^M function. Compared to group-housed wild-type antennae, social isolation has the least number of significantly altered genes, whereas group-housed *fru^M* mutants resulted in the highest number of significantly differential gene expression (Figure 2A-C). Pairwise comparisons of social deprivation and lack of pheromone receptors/Fru^M function demonstrate the social experience/Or47b/Or67d/Fru^M-dependent transcriptional programs, and notably reveal that the genes co-regulated by pheromone receptors and Fru^M tend to behave in the same direction in the corresponding mutants (Figure 2A,D), suggesting the shared downstream signaling pathways upon pheromone receptor activation and Fru^M-dependent regulation. The numbers of genes with significant differential expression in the same direction shared by each condition compared to the group-housed wild types are illustrated in a Venn diagram and Upset plot (Figure 2B,C), where genes with overlapping changes in social isolation and *Or47b*, *Or67d*, and *fru^M* mutants are highlighted. Particularly, only one gene, *CG13659*, an ecdysteroid kinase-like domain encoding gene, is consistently changed across all experimental conditions compared to antennae from the group-housed wild-type males (Figure 2B).

Hierarchical cluster analysis of $\log_2$ fold change versus group-housed wild-type samples shows changes in *fru^M* and *Or67d* mutants as being the most comparable, with single-housed wild types being an outlier of the four groups (Figure 2E). We found that no cluster has a specific bias towards low, medium, or high expression of genes (Figure 2E). Cluster analysis identified several genes of behavioral, neuromodulatory, and developmental importance such as *Cytochrome p450 6a20* (*Cyp6a20*)*, serotonin receptor 2A* (*5-HT2A*)*, Juvenile hormone esterase* (*Jhe*)*,* and *Dpr-interacting protein alpha* (*DIP-alpha*) (Figure 2E) (Carrillo et al., 2015; Johnson et al., 2009; Liu et al., 2008; Wang et al., 2008)*.* Among these, antennal expression of *Cyp6a20,* which is downregulated in *Or47b*, *Or67d*, and *fru^M* mutants, was previously shown to mediate effects of social experience on male-male aggression (Figure 2E) (Wang et al., 2008). On the

other hand, *Cyp4p2,* which is involved in hormone metabolism and insecticide detoxification (Scanlan et al., 2020; Seong et al., 2019, 2018), is only misregulated in *Or47b* mutants (Figure 2E). In addition to the downregulated genes, we also found some genes encoding ion channels and neurotransmitter receptors that show significant upregulation (*ppk25* and *GluRIIa*) (Figure 2E). The heatmap for gene expression changes revealed gene clusters that are co-regulated by pheromone receptors and Fru^M, in addition to gene clusters that are uniquely regulated by each OR and Fru^M; this again highlights that the co-regulated genes tend to change in the same direction in pheromone receptor and fru^M mutants.

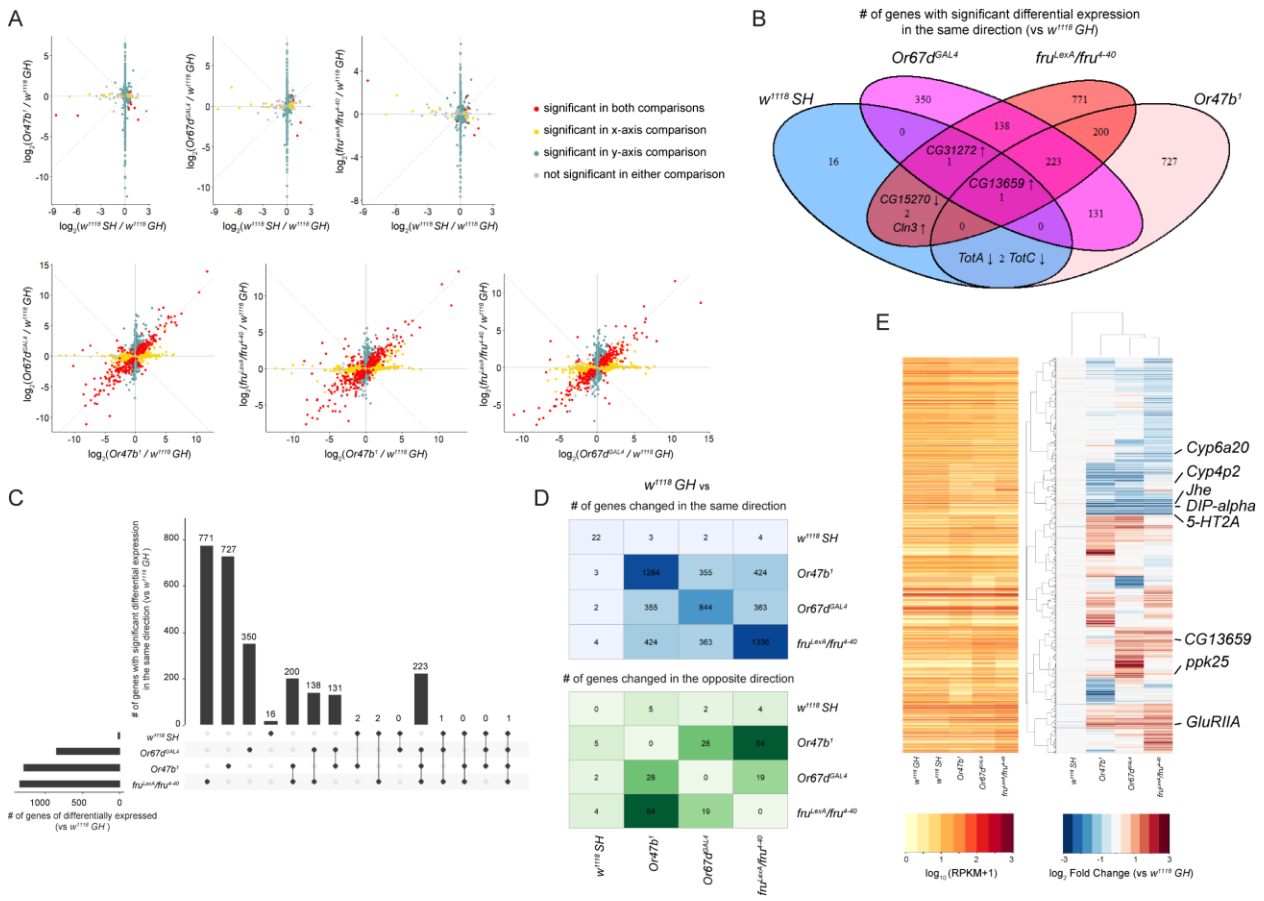

Figure 2. Differentially expressed genes in response to loss of social experience, pheromone receptors, or *fru*^{M}. (A) Scatter plot showing the genes that are differentially regulated among social isolation, and mutants in pheromone receptors and *fru*^{M}. Significance is defined by adjusted p-value below 0.01 after applying Bonferroni correction with n = 2. **(B-C)** Venn diagram **(B)** and UpSet plot **(C)** comparing differentially expressed genes shared across experimental conditions (only genes changed in the same direction). **(D)** Numbers of differentially expressed genes with the same (top) and the opposite (bottom) direction in pairwise comparison of experimental conditions versus group-housed wild-type samples. In (B-D), significance is defined by adjusted p-value below 0.01 after applying Bonferroni correction with n = 4. **(E)** Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-housed wild-type antennae across all experimental conditions (right) and average mRNA levels (reads per kilobase of transcript, per million mapped reads, RPKM) of replicates within each

condition ordered in the same way as $\log_2$ fold change (left). Only 2999 genes with at least one significant (adjusted p-value below 0.01) change between an experimental condition versus group-housed wild types are shown.

Gene ontology terms for differentially expressed genes in response to lack of social and pheromone signaling highlight neuromodulators

Previous work has demonstrated that social experience, pheromone signaling, and Fru^M activity can regulate the responsiveness of pheromone sensing ORNs to modify circuit function and behavior (Dweck et al., 2015; Kurtovic et al., 2007; Lin et al., 2016; Liu et al., 2011; Sethi et al., 2019; Wang et al., 2008). To functionally understand system-level changes in gene expression with social isolation, lack of pheromone signaling, and *fru^M* mutants, we next investigated gene ontology (GO) terms using GOrilla for the list of differentially expressed genes in each experimental condition in pairwise comparisons with group-housed wild types (Eden et al., 2009, 2007) (Figure 3). Many GO terms of molecular function and biological process are commonly affected across multiple experimental groups, suggesting the converging downstream molecular events in response to social experience and pheromone sensing mediated by Fru^M activity (Figure 3). Strikingly, the genes with the altered expression tend to be localized on the cell membrane (Figure 3, GO: cellular component) and have functions in ion transport across membrane (Figure 3, GO: molecular function), and appear to be involved in the process of detecting and responding to olfactory stimuli (Figure 3, GO: biological process). This supports previous studies in providing a general mechanism for social experience, pheromone receptor signaling, and Fru^M-dependent regulation of pheromone responsiveness of Or47b ORNs (Sethi et al., 2019; Zhang et al., 2020; Zhao et al., 2020). Furthermore, genes with oxidoreductase activity also have overlapping alterations across *Or47b*, *Or67d,* and *fru^M* mutants, and many of these appear to contribute to insect hormone metabolism (Figure 3, GO: molecular function). Interestingly, previously studies reported that juvenile hormone signaling works together with social experience in olfactory receptor neurons to modulate chromatin around *fru* locus (Sethi et al., 2019; Zhao et al., 2020). Our RNA-seq results also add an additional layer of complexity to hormone-social experience interactions, as social experience

and pheromone signaling affects the levels of certain hormones by modifying hormone metabolism dynamics. In summary, social isolation, disrupted pheromone receptor signaling, and lack of Fru^M function in peripheral olfactory sensory neurons affect the expression of many genes with roles in diverse aspects of neurophysiology, including neuronal responsiveness, ion transmembrane transport, and beyond.

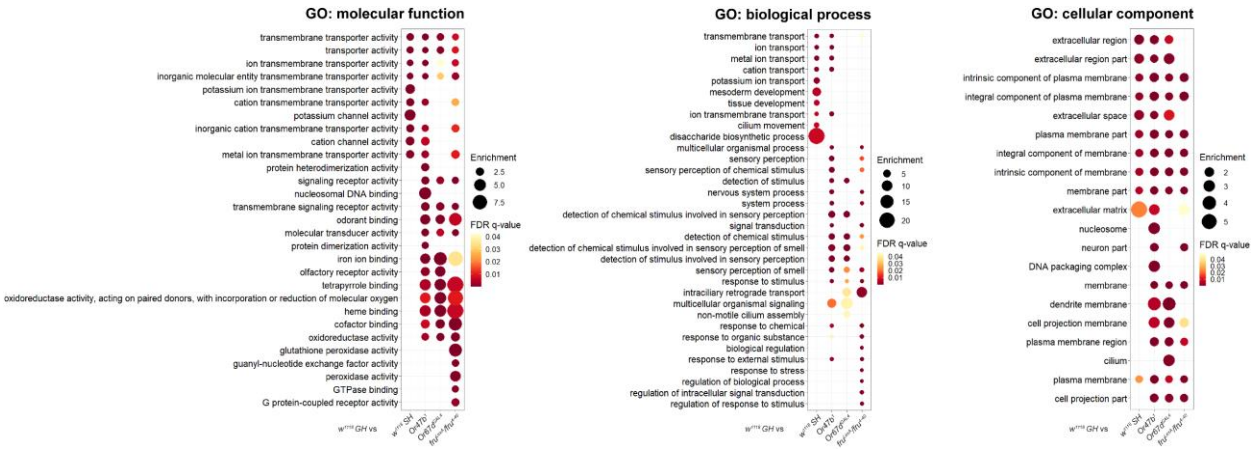

Figure 3. Top enriched gene ontology (GO) terms for differentially expressed genes in response to social experience, pheromone signaling, and Fru^{M} activity. The union set of top 10 most significantly enriched GO terms with FDR q-value below 0.05 of the differentially expressed genes in each experimental condition is shown. Enriched GO terms were generated by the single ranked gene list with the smallest adjusted p-value at the top via GOrilla.

Loss of pheromone signaling alters *fruitless* splicing patterns

Fruitless locus generates multiple alternative splice isoforms for RNAs transcribed from 7 promoters (P1-P6, PD). The transcripts from *fru P1* are alternatively spliced between males and females, where the male, but not the female, isoform (fru^M) encodes functional proteins (Dickson, 2008; Yamamoto and Koganezawa, 2013). The expression of fru^M in males and the absence of functional fru^F transcripts in females help define male and female-specific neuronal pathways as well as the cell-specific expression patterns of genes regulated by Fru^M. Promoter *fru P2* through *fru P6* produce common isoforms in both males and females that also affect sex-specific activity in courtship circuits of both sexes (Goodwin et al., 2000) (Figure 4A). In addition, special transcripts from *fru PD* and *fru P4* produce a less studied common transcript 3' exon (region 16, exon D) (Figure 4A) as well as a potential regulatory transcript lacking a DNA bind domain and large 3' UTR (region 18, exon C5 in *fru-RA* transcript) (Usui-Aoki et al., 2000) (Figure 4A). Fru^M itself has multiple splicing isoforms that vary in the 3' end of the mRNA (fru^{MA}, fru^{MB}, fru^{MC}), which encode proteins with variable zinc finger DNA binding domain of Fru^M transcription factor (Goodwin et al., 2000; Nojima et al., 2014; Vernes, 2014). These regulate different aspects of the circuit controlling courtship behaviors, with Fru^{MC} and Fru^{MB} having the highest overlap behaviorally, and Fru^{MA} having little to no effect on courtship (Neville et al., 2014).

We previously showed that social experience and signaling from Or47b and Or67d pheromone receptors alter open chromatin marks around *fru P1* promoter in the male antennae (Zhao et al., 2020). Interestingly, examination of total transcript levels for the entire *fru* gene locus showed little to no difference across experimental conditions (Figure 1D). These small changes in total transcript levels, despite dramatic changes in open chromatin marks in wild-type SH, and mutants in *Or47b*, *Or67d,* and fru^M, prompted us to look at other aspects of gene regulation. It is known that changes in chromatin regulate many aspects of transcription such as transcriptional

initiation, elongation and alternative splicing (Adam and T., 2011; Naftelberg et al., 2015). The effects of chromatin on splicing are thought to occur especially because chromatin state alters the speed of RNA Polymerase II (RNAPII), which can lead to splicing mistakes like intron retention or exon skipping (Adam and T., 2011).

Given the functional differences in the *fru*^{M} isoforms, we predicted that chromatin changes caused by social experience and pheromone receptor signaling could alter *fru* splicing. To explore this, we mapped reads from all experimental conditions to *fru* genomic locus and investigated exon levels using DEXseq (Anders et al., 2012). In general, transcript reads from *fru* locus appear noisier in experimental conditions compared to group-housed wild-type male antennae, with variations in the expression of coding and non-coding sequences (Figure 4B-E). In *Or47b* mutants, there is a small decrease in exon 1 (*fru P1* promoter) and male-specific exon (exon 2) levels (Figure 4C; see methods – statistical analysis). *Or67d* mutants show a small decrease in exon 1 levels, but male-specific exon (exon 2) is unchanged (Figure 4D). The largest change in male-specific exon levels is seen in *fru*^{LexA}/*fru*$^{4-40}$ allele (Figure 4B), which has a *LexA* transgene insertion disrupting the male-specific exon and a 70-Kb deletion from the *P1* promoter (Mellert et al., 2010). Exon 1 and the male-specific exon are unaltered in socially isolated antennae, yet there is a small increase in the female-specific exon (exon 3) (Figure 4E). In addition to the first 3 exons, a non-coding sequence (region 18) (Figure 4A), which is a part of *fru-RA* transcript (Usui-Aoki et al., 2000), significantly increases in *Or67d, Or47b*, and *fru*^{M} mutants (Figure 4C,D). This transcript encodes a Fru protein that lacks these zinc finger domains but retains BTB/PDZ protein-protein interaction domain. Quantitative reverse transcription PCR (qRT-PCR) experiments confirmed this finding (Figure 4-figure supplement 1). Increases in other untranslated regions (regions 20 and 22) are also observed in mutant samples. These results suggest social and pheromonal cues alter *fru* exon usage, likely indicating changes in *fru* splicing patterns.

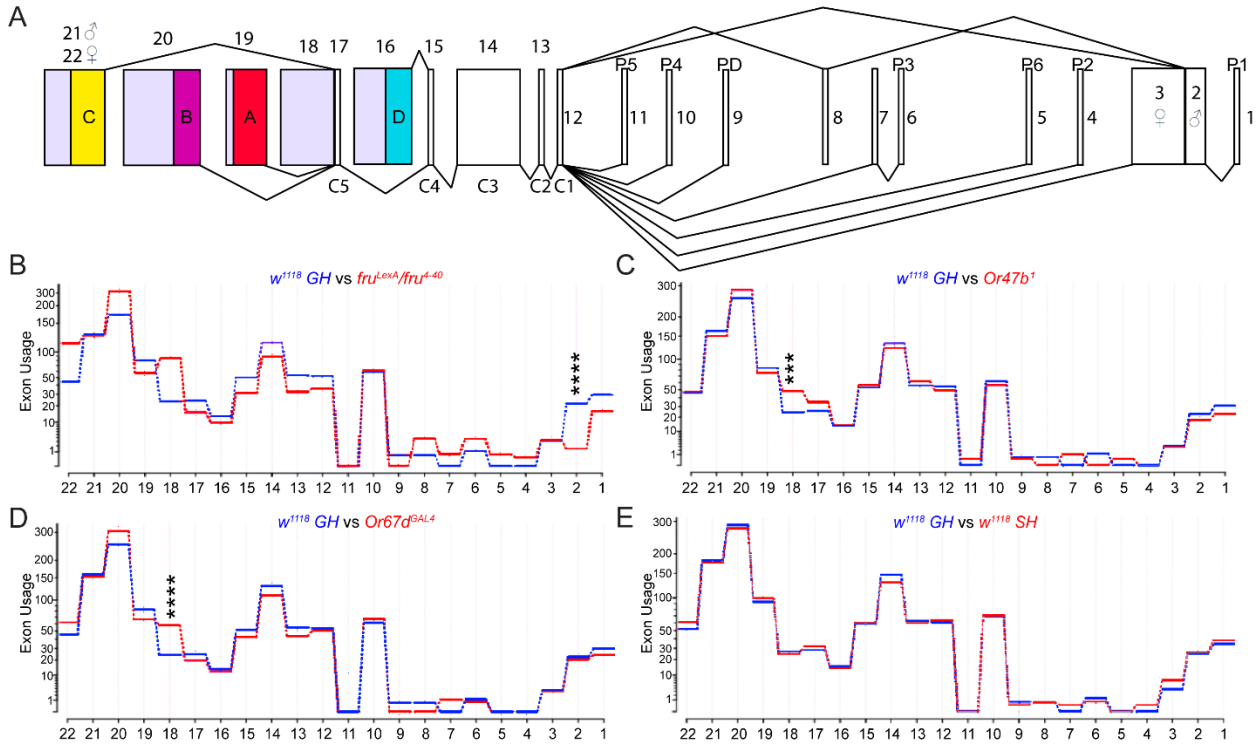

Figure 4. Olfactory stimuli regulate exon usage across the whole *fru* genomic locus. Examination of the usage of various exons in Fruitless to determine distinct changes in fru^{M} transcripts using DEXSeq. **(A)** Schematic of Fruitless: 1-22 denote each examined site. P1-P6 and PD denote promoters. C1-C5 denote common exons, while A-D denote alternatively spliced 3' DNA binding encoding domains. **(B-E)** Exon-by-exon comparison of w^{1118} *GH* vs w^{1118} *SH* **(B)**, $Or47b^{1}$ **(C)**, $Or67d^{GAL4}$ **(D)**, and fru^{LexA}/fru^{4-40} **(E)**. Adjusted p-value was directly performed via DEXSeq (see methods – statistical analysis). ***, p.adjust<0.001; ****, p.adjust<0.0001. Exon 2, w^{1118} *GH* vs fru^{LexA}/fru^{4-40}, p.adjust=1.25×10^{-10}; Exon 18 (3'UTR), w^{1118} *GH* vs $Or47b^{1}$, p.adjust=8.17×10^{-4}; Exon 18 (3'UTR), w^{1118} *GH* vs $Or67d^{GAL4}$, p.adjust=2.00×10^{-7}.

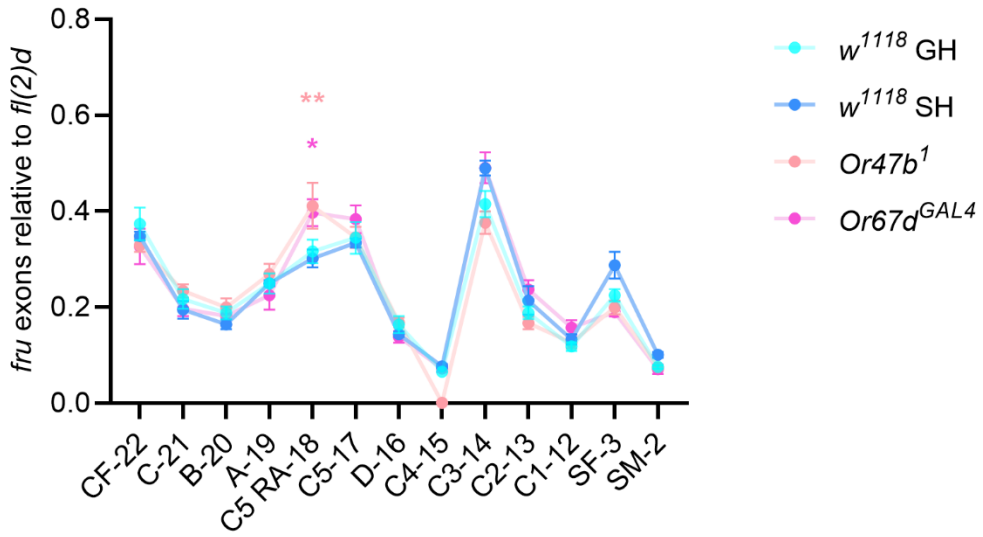

Figure 4-figure supplement 1. Quantitative RT-PCR of *fru* exons from antennae of group-housed and single-housed wild types, *Or47b* mutants, and *Or67d* mutants. For each genotype or condition, n = 4 biological replicates. Two-way ANOVA followed by multiple comparisons within each exon (compare w^{1118} *SH*, *Or47b* mutants, and *Or67d* mutants to w^{1118} *GH*). *, p<0.05; **, p<0.01. C5 RA (exon 18): $Or47b^{1}$ vs w^{1118} *GH* (p=0.0023), $Or67d^{GAL4}$ vs w^{1118} *GH* (p= 0.0123).

Bimodal regulation of genes modulating neurophysiology and neurotransmission by Fru^M and pheromone receptor signaling

Previous studies have shown that pheromone receptor signaling and social experience-dependent regulation of chromatin and RNAPII enrichment around *fru P1* promoter can ultimately scale and fine-tune behavioral responses to social environment (Sethi et al., 2019; Zhao et al., 2020). Additionally, previous reports on the genome-wide binding profiles for three Fru^M isoforms in the central brain revealed isoform-specific differences in target genes that regulate neuronal development and function (Billeter et al., 2006; Neville et al., 2014). Fru^M motifs are enriched among regulatory elements that are open in the female but closed in the male, suggesting Fru^M functions as possible repressive transcription factor (Brovkina et al., 2021). Functional differences of Fru^M isoforms also influence ORN responses to their pheromone ligands (Zhang et al., 2020). Thus, chromatin-based modulation of *fru* levels and splicing with social experience and pheromone signaling can provide a quick way to modulate neuronal physiology and synaptic communication by modifying gene expression programs. Yet, the effect of social experience and pheromone receptor signaling on gene expression programs or the mode of gene regulation by Fru^M (as a transcriptional activator, repressor, or both) remains unknown.

As discussed previously, gene ontology analysis of these differentially expressed genes implies that many genes involved in neuromodulation are regulated by social context, pheromone receptor signaling, and Fru^M function. To further investigate this, we specifically zoomed into genes associated with ion channel activity and/or neurotransmitter regulation (Figure 5A,B; Figure 6A,B). We clustered these genes based on their log_2 fold change in transcript levels compared to group-housed wild types in each experimental condition, while also showing their corresponding expression levels in the antennae (RPKM, reads per kilobase of transcript, per million mapped reads) (Figure 5A,B; Figure 6A,B). We found many ion channel and/or

neurotransmitter receptor-encoding genes showed up/down-regulation in response to social isolation, and loss of Or47b, Or67d, or Fru^M function (Figure 5A,B; Figure 6A,B). Within ion channels, two subclasses stand out. These are the Degenerin/Epithelial Sodium Channel (DEG/ENaC) proteins known as pickpockets (ppks) and inward-rectifying potassium channels Irks. Additional genes also include those encoding calcium channels, for example, *Piezo*, *TrpA1*, and *cacophony* (*cac)* (Figure 5A,B)*.*

<u>ppk gene family</u>

Recent reports pointed to the function of DEG/ENaC channels known as *pickpocket* family of sodium channels that act in Or47b and Or67d ORNs to regulate responses to their ligands (Zhang et al., 2020). Fru^M binding motifs have been identified around many of these *ppk* family members, such as *ppk*, *ppk5*, *ppk6, ppk15, ppk19*, *ppk23*, *ppk25,* and *ppk30* (Dalton et al., 2013; Neville et al., 2014; Vernes, 2014). Both *ppk23* and *ppk25* have been identified as necessary for changes of physiology in Or47b ORNs through Fru^{MB} and Fru^{MC} activity, respectively, with Fru^{MB} having an antagonistic effect on physiology in Or67d ORNs (Zhang et al., 2020). We therefore first examined the expression levels of *ppk* genes in the antennae. In group-housed wild-type antennae, *ppks* show varying degrees of and generally low expression, with *ppk5* displaying the highest levels (Figure 5C). Many *ppk* genes, including *ppk23,* seem not to be expressed in antenna. Analysis of recent single-cell RNA-seq data from ORNs (Li et al., 2020) revealing ORN class-specific expression of *ppk*s also agree with this pattern, though a few *ppks* known to be expressed in several ORN classes are not detectable in this dataset, possibly due to the limitations of single-cell RNA-seq in detecting low-abundance genes (Figure 5-figure supplement 1A). Even though there is no effect on the expression of any *ppk* family members in socially isolated male antennae, many *ppk* genes are differentially regulated in fru^M mutants, in agreement with the existing Fru^M binding sites at their promoters. For example*, ppk6 and ppk25* are upregulated in fru^M mutants whereas *ppk5*,*7*,*13*,*14*,*15*,*19* are downregulated.

Many of these genes also show correlated changes in *Or47b* and/or *Or67d* mutants (*ppk13*,*14*,*15*,*19*,*25*). *ppk6* is strikingly upregulated in both *fru*^{M} and *Or67d* mutants, whereas *ppk7* is downregulated in both *Or47b* and *fru*^{M} mutants (Figure 5C'). Of note is the significant changes in *ppk25* expression as *ppk25* has been found to change the sensitivity of Or47b neurons, is expressed in Or47b and Ir84 ORNs, but not Or67d ORNs, and has recently been shown to be downstream of Or47b and Ir84a activity (Ng et al., 2019; Pikielny, 2012) (Figure 5C). The changes in *ppk25* levels were also validated through quantitative RT-PCR (Figure 5-figure supplement 1C-E). The bimodal changes in *ppk* transcripts in *fru*^{M} mutants suggest Fru^{M} can act as both a repressor and an activator of *ppk* gene regulation. Furthermore, given that pheromone receptor function regulates Fru, the patterns of *ppk* gene misregulation in *Or47b* and *Or67d* mutants are likely dependent on changes in Fru^{M} activity downstream of pheromone receptor signaling.

<u>Irk gene family</u>

Irk gene family encodes 3 inwardly rectifying potassium channels (Irk1-3) with binding motifs for Fru^{MA} identified upstream of *Irk2* and binding of both Fru^{MA} and Fru^{MC} found around *Irk3* (Dalton et al., 2013; Neville et al., 2014; Vernes, 2014). Three *Irk* genes are expressed in varying levels in the antennae with *Irk1* having the lowest expression and *Irk2* having the highest expression (Figure 5D). We found that *Irk1* is upregulated in *Or47b* mutants, whereas *Irk2* shows the trend towards upregulation in response to social isolation (Figure 5D').

These results suggest that changes in the transcript levels of Fru^{M}-regulated sodium and potassium channels with social isolation and in pheromone receptor mutants may contribute to changes in neuronal responses and behaviors.

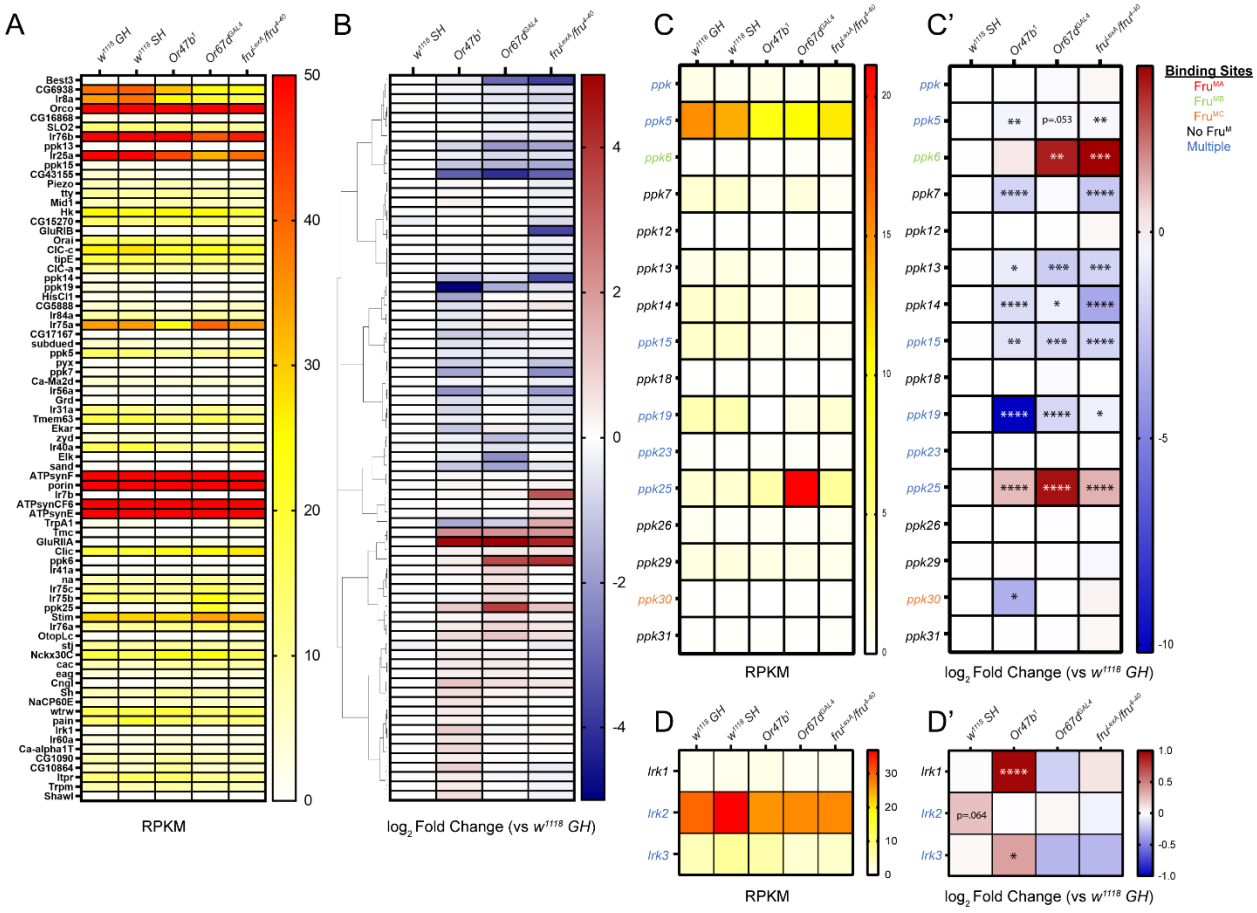

Figure 5. Differentially expressed ion channel-encoding genes in response to social isolation and loss of pheromone receptors or *fru*^{M}. (A-B) Examination of GO term: 0005216 (ion channel activity) shows significant changes in various ion channel subclasses. Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-housed wild-type antennae across all experimental conditions **(B)** and average mRNA levels (RPKM) of replicates within each condition ordered in the same way as $\log_2$ fold change **(A)**. Genes that are not significant (adjusted p-value above 0.01) in any experimental condition were filtered out. **(C-C')** RPKM **(C)** and $\log_2$ fold change **(C')** for *pickpocket* (*ppk*) gene family. **(D-D')** RPKM **(D)** and $\log_2$ fold change **(D')** for *inwardly rectifying potassium channel* (*Irk*) gene family. Adjusted p-value was directly performed via DESeq2. *, p.adjust<0.05; **, p.adjust<0.01; ***, p.adjust<0.001; ****, p.adjust<0.0001. FruM binding information is listed in the supplementary file.

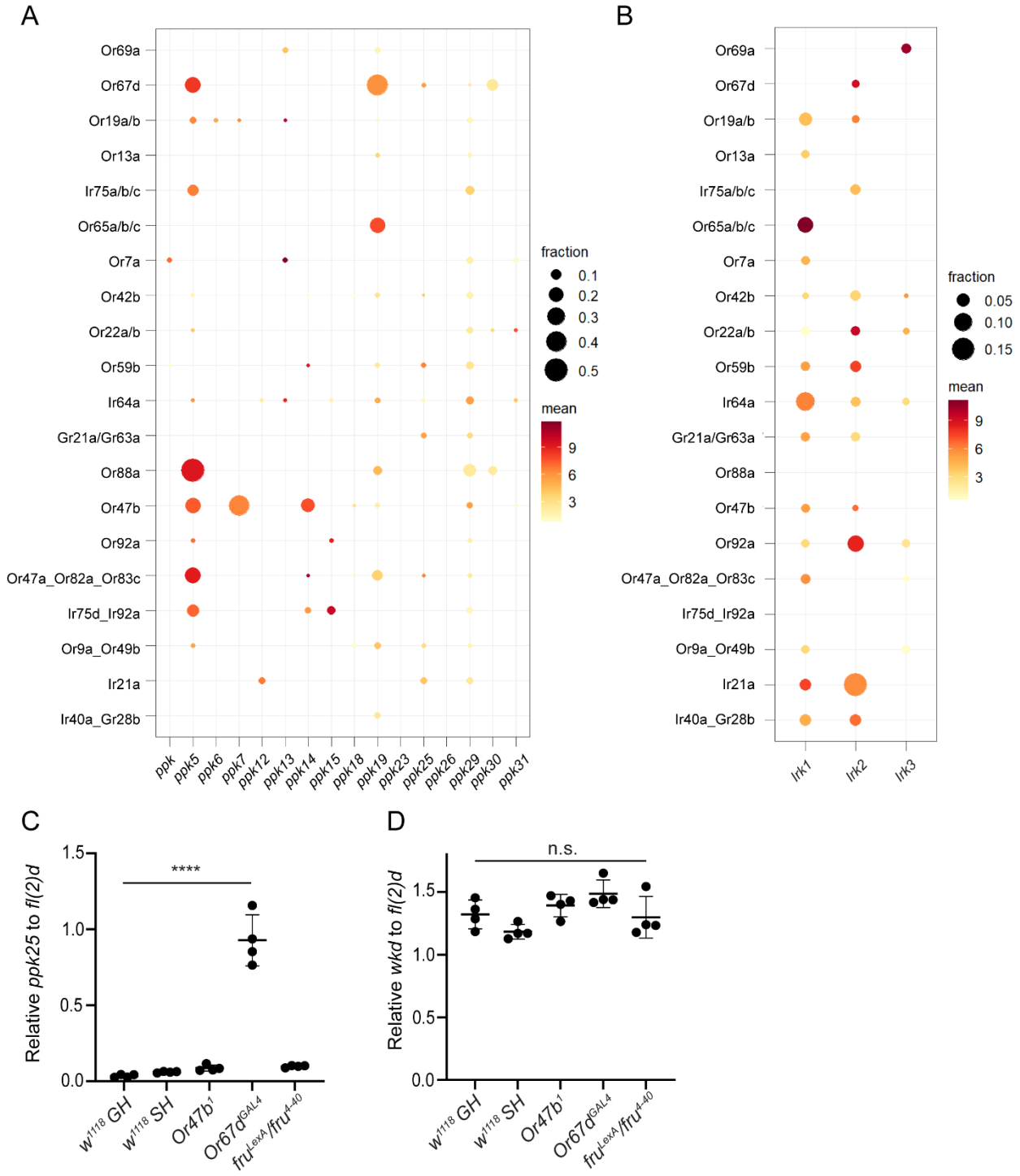

Figure 5-figure supplement 1. Validation of *ppk* expression across ORN classes and in mutants. (A-B) ORN class-specific expression of *ppk* and *Irk* family genes based on single-cell RNA-seq datasets from the adult ORNs (McLaughlin et al., 2021)**.** Size of each circle indicates the fraction of positive cells ($\log_2$(CPM+1)>0.5) and color intensity indicates the mean

expression ($\log_2$(CPM+1)) of all positive cells. **(C-D)** Quantitative RT-PCR validation of *ppk25* expression (C) and a negative control gene *wkd* expression (D) from antennae of grouped and socially isolated wild types, *Or47b* mutants, *Or67d* mutants, and fru^M mutants normalized to *fl(2)d*. *fl(2)d* and *wkd* were selected based on their near-identical expression level across all conditions from the RNA-seq results and thus used as loading and negative control genes. For each genotype or condition, n=4 biological replicates. One-way ANOVA followed by multiple comparisons (compare other groups to group-housed wild types w^{1118} *GH*). ****, $p<0.0001$.

Regulators of Neurotransmission

To ask if social experience, pheromone signaling, and Fru^M function regulate genes involved in neurotransmission, we next examined the expression of neurotransmitter receptors, transporters, and synthesizers. ORNs in the antennae are mostly cholinergic (Wilson, 2013). In the antennal lobe it has been shown that lateral neurons, which include serotonergic, GABAergic, and glutamatergic interneurons, provide cross talk between antennal lobe neurons (Chou et al., 2010; Wilson, 2013). These neurons form connections on the presynaptic bouton of ORN synapses to cause lateral excitation or inhibition across glomeruli (Olsen et al., 2007; Wang et al., 2003; Wilson, 2013). These connections are required for fine tuning of signaling at synapses as a way of rapid modulation of neuronal function (Dacks et al., 2009; Johnson et al., 2009; Mohamed et al., 2019; Olsen et al., 2007; Sizemore and Dacks, 2016; Sudhakaran et al., 2012; Suzuki et al., 2020; Wang et al., 2003; Wong et al., 2002; Zhang et al., 2019). We found a high expression of choline acetyltransferase (*ChAT*) that catalyzes acetylcholine biosynthesis and *VAChT* that packages acetylcholine into synaptic vesicles, coinciding with their reported cholinergic roles. Moreover, we also found relatively high expression of several genes encoding receptors of various neurotransmitters, such as choline, serotonin (5-HT), GABA, and glutamate (Figure 6C-F'). Many of these genes, such as *nAChRalpha4*/*5*, *5-HT2A*, *5-HT7*, *GABA-B-R2*, and *GluRIIA*, have previously been found to regulate courtship behavior in flies through signaling in the antennal lobe (Becnel et al., 2011; Clowney et al., 2015; Johnson et al., 2011; Suzuki et al., 2020). Interestingly, *GABA-B-R2* was shown to be specifically involved in presynaptic gain control of Or47b ORNs (Root et al., 2008). Additionally, single-cell RNA-seq data shows both broadly expressed neurotransmitter genes like *GluRIIB* and 5-*HT2B*, while others are specific to a subset of ORN classes (McLaughlin et al., 2021) (Figure 6-figure supplement 1). Overall, many of the genes encoding neurotransmitter receptors show expression changes in different experimental conditions (Figure 6B).

To zoom into genes related to specific neurotransmitters, we didn't observe any significant changes in response to social isolation, except a few genes, like *dmGlut,* appear to be upregulated compared to the grouping condition (Figure 6C,D'). We again found that loss of Fru^M function led to bimodal effects on gene expression (Figure 6C-F'). Indeed, many of these genes have known Fru^M binding to their promoters, including receptors *nAChRalpha1*/*3*/*4*/*5,* G*luRIIA*, *GluClalpha*, *5-HT1A*, *5-HT1B*, *5-HT2A*, *5-HT7*, and transporters/regulators such as *VAChT, ChAT*, and *Gat* (Dalton et al., 2013; Neville et al., 2014; Vernes, 2014). Some of these genes display correlated changes between pheromone receptor mutants and fru^M mutants, like *GluRIIA*, *dmGlut*, and *5-HT2A*, suggesting that the effects of pheromone signaling on neurotransmission can act via their influences on *fru* regulation (Figure 6D-E'). The changes in *5-HT2A* were also validated through qRT-PCR (Figure 6-figure supplement 2). Evident changes are also observed in some genes not known to be Fru^M targets, for example, *GluRIB* which shows downregulation only in fru^M mutants, and *5-HT2B* which shows upregulation in *Or47b* and fru^M mutants (Figure 6D-E'). These may reflect effects of pheromone receptor signaling independent of Fru^M function or indirect effects of Fru^M activity. To summarize, the systems-level changes in expression of genes involved in neurotransmission and neurophysiology with social experience and pheromone receptor signaling can modulate ORN responses. In addition, these effects on gene expression with social signals can occur either in a Fru^M-dependent manner or independently of Fru^M in response to other gene regulatory pathways activated by pheromone receptor signaling.

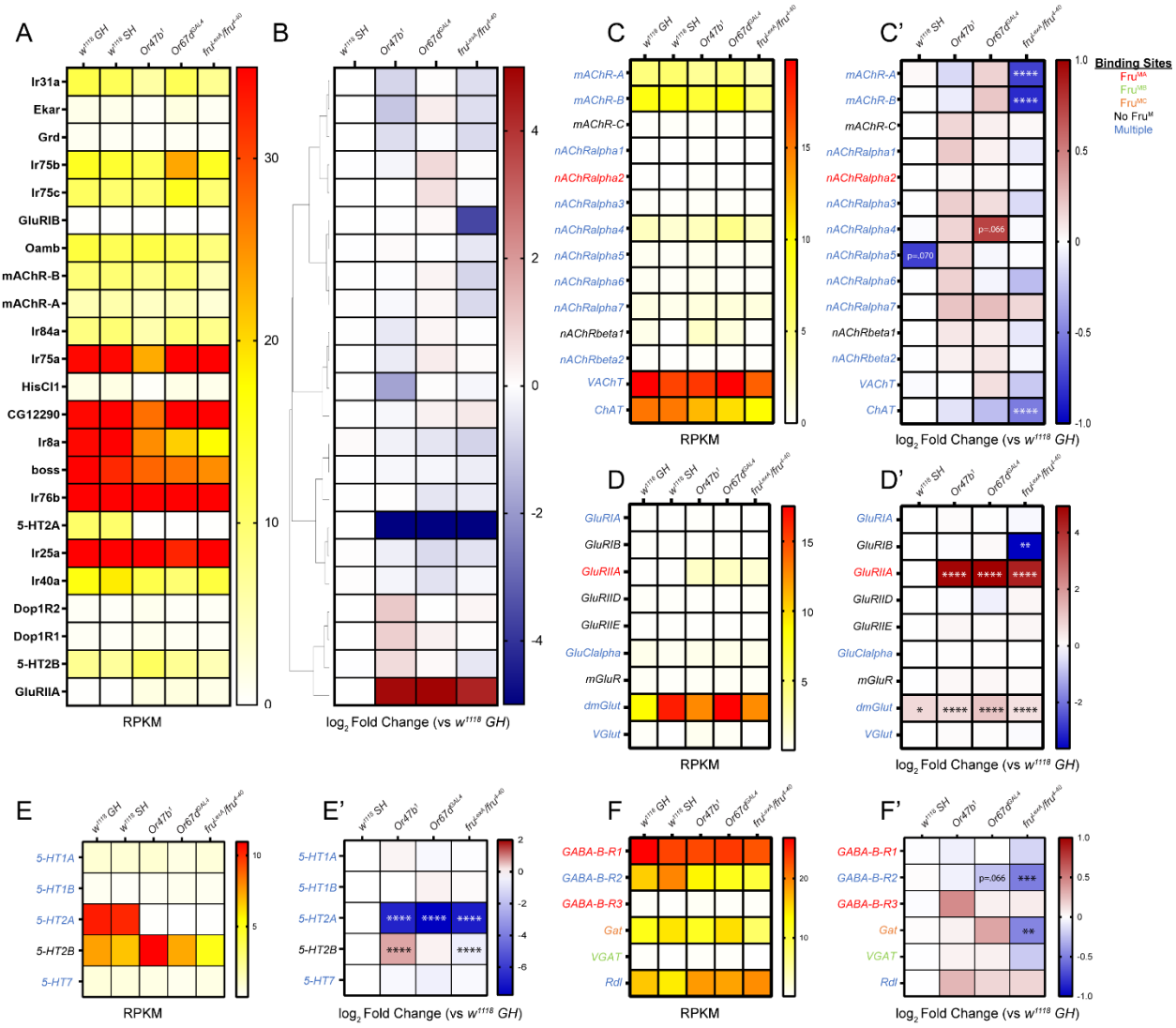

Figure 6. Differentially expressed neurotransmitter receptor and transporter-encoding genes in response to social isolation and loss of pheromone receptors or *fru*^{M}. (A-B) Examination of GO term: 0030594 (neurotransmitter receptor activity) shows significant changes in various neurotransmitter activity-associated subclasses. Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-housed wild-type antennae across all experimental conditions **(B)** and average mRNA levels (RPKM) of replicates within each condition ordered in the same way as $\log_2$ fold change **(A)**. Genes that are not significant (adjusted p-value above 0.01) in any experimental condition were filtered out. **(C-C')** RPKM **(C)** and $\log_2$ fold change **(C')** for acetylcholine-associated genes. **(D-D')** RPKM **(D)** and $\log_2$ fold

change **(D')** for glutamate-associated genes. **(E-E')** RPKM **(E)** and log_2 fold change **(E')** for serotonin-associated genes. **(F-F')** RPKM **(F)** and log_2 fold change **(F')** for GABA-associated genes. Adjusted p-value was directly performed via DESeq2. *, p.adjust<0.05; **, p.adjust<0.01; ***, p.adjust<0.001; ****, p.adjust<0.0001. FruM binding information is listed in the supplementary file.

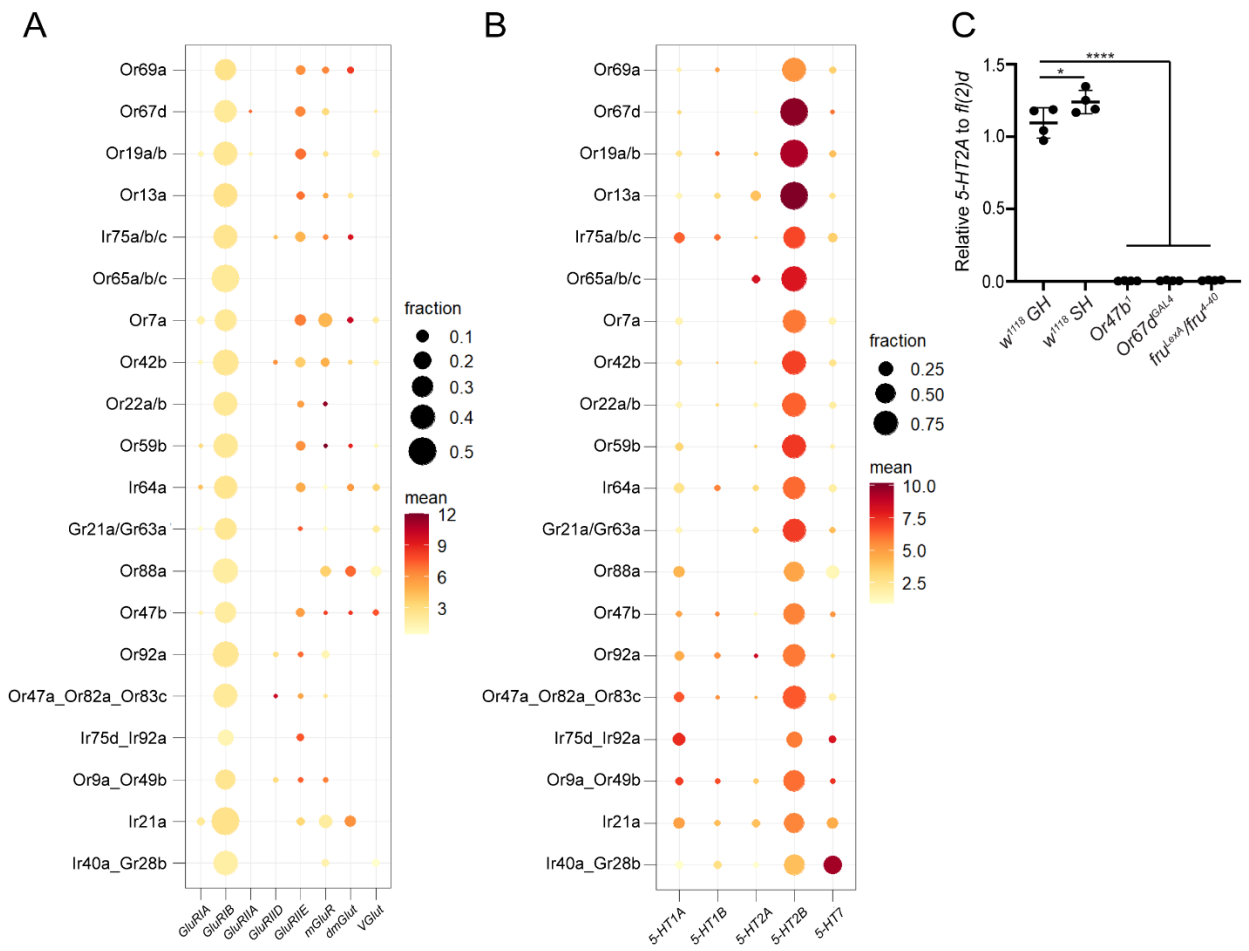

Figure 6-figure supplement 1. Validation of neurotransmitter receptor expression across ORN classes and in mutants. (A-B) ORN class-specific expression of serotonin and glutamate receptors based on single-cell RNA-seq datasets from the adult ORNs (McLaughlin et al., 2021)**.** The size of each circle indicates the fraction of positive cells ($\log_2$(CPM+1)>0.5) and color intensity indicates the mean expression ($\log_2$(CPM+1)) of all positive cells. **(C)** Quantitative RT-PCR validation of *5-HT2A* expression. Relative expression of *5-HT2A* from antennae of grouped and socially isolated wild types, *Or47b* mutants, *Or67d* mutants, and fru^M mutants normalized to *fl(2)d*. For each genotype or condition, n=4 biological replicates. One-way ANOVA followed by multiple comparisons (compare other groups to group-housed wild types w^{1118} *GH*). *, p<0.05; ****, p<0.0001.

Pheromone receptor signaling regulates genes involved in hormone metabolism

Previous studies have identified age-related cues such as juvenile hormone (JH) signaling together with social experience to control Or47b neuronal responses to pheromones and courtship behaviors in a Fru^M-dependent manner (Lin et al., 2016; Sethi et al., 2019; Zhang and Su, 2020). JH signaling, concurrent with social experience, modifies chromatin around *fru P1* promoter and ultimately fru^M levels in Or47b ORNs (Zhao et al., 2020). These studies also demonstrated that JH receptor enrichment at *fru P1* promoter increases in socially isolated flies as well as flies with disrupted Or47b signaling (Zhao et al., 2020). As mentioned above, gene ontology analysis in this study also brings genes involved in hormone metabolism (Figure 3). Thus, we specifically interrogated the genes regulating hormone levels in pheromone receptor and fru^M mutants (Figure 7A-C').

Many of the enzymes involved in juvenile hormone biosynthesis and metabolism, such as juvenile hormone epoxide hydrolases (*Jheh1*,*2*,*3*), juvenile hormone esterase (*Jhe*), and juvenile hormone acid methyltransferase (*jhamt*), are expressed at varying levels in the antennae (Figure 7C; Figure 7-figure supplement 1). These genes are also reported to have Fru^{MA} and Fru^{MC} binding in their upstream regulatory elements (Dalton et al., 2013; Neville et al., 2014; Vernes, 2014). Two mostly enriched genes, *Jheh1* and *Jheh2*, show mild upregulation in fru^M mutants but no significant changes in the absence of social cue or pheromone receptor signaling (Figure 7C,C'). On the other hand, both *Jhe* and *Jheh3* appear to be upregulated in social isolation while downregulated in *Or47b* mutants (Figure 7C'; Figure 7- figure supplement 1). *Jhe* is of particular interest as Jhe activity is known to be necessary for robust male-specific courtship behaviors and mating success in addition to affecting the abundance of sex-specific pheromones such as 11-cis-vaccenyl acetate in males (Ellis and Carney, 2010; Liu et al., 2008). Furthermore, seminal work on Jhe and Jheh3 has shown that these enzymes work together to

catabolize JH in *D. melanogaster* (Khlebodarova et al., 1996). These results suggest that social experience and pheromone receptor signaling regulates the expression of JH biosynthetic enzymes. Such changes can modulate juvenile hormone activity by rapidly catabolizing JH in the periphery and affecting downstream target genes, such as *fruitless*.

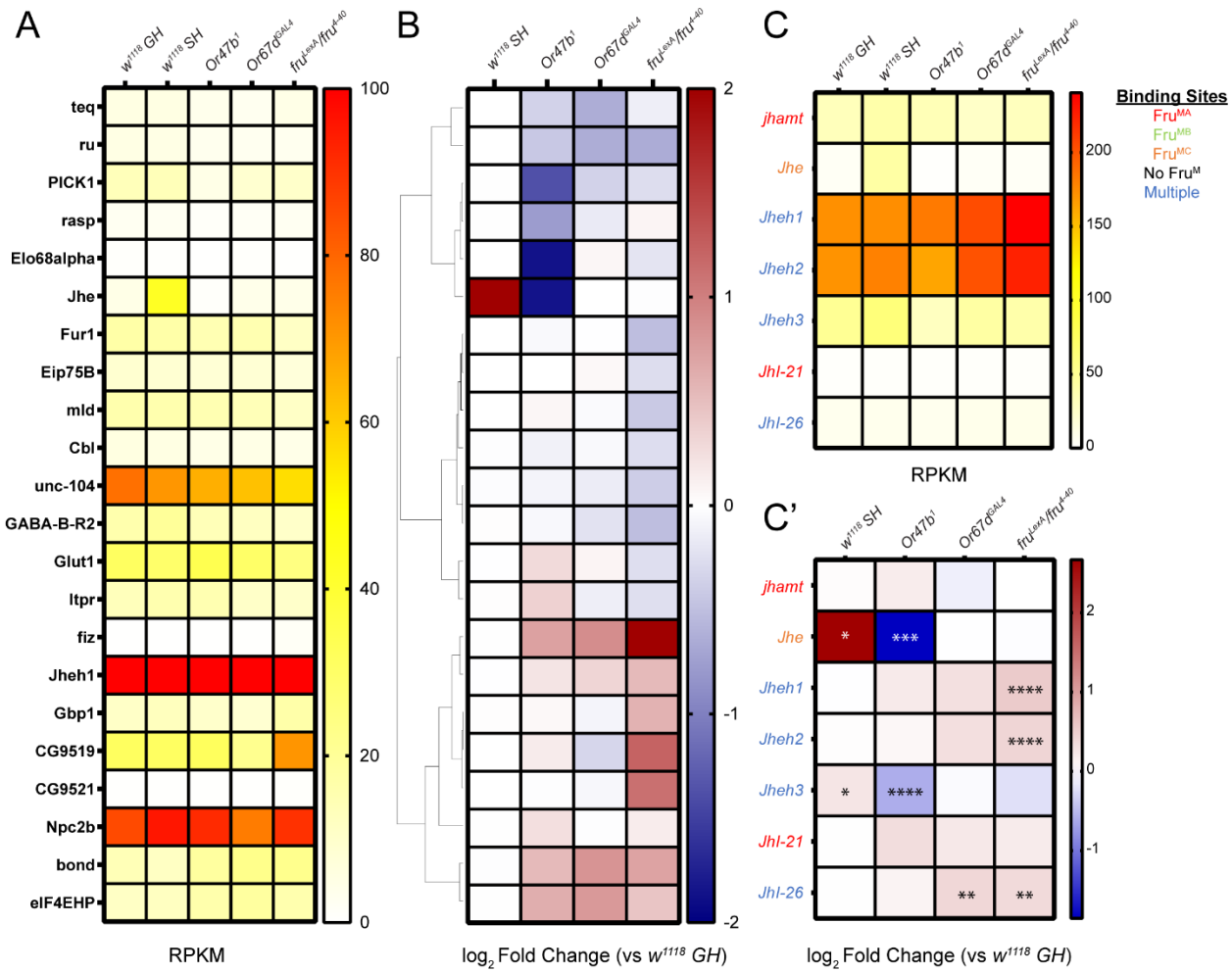

Figure 7. Differentially expressed hormone regulator-encoding genes in response to social isolation and loss of pheromone receptor or *fru^{M}*. (A-B) Examination of GO term: 0010817 (regulation of hormone levels) shows significant changes in various hormone regulation-related gene subclasses. Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-housed wild-type antennae across all experimental conditions **(B)** and average mRNA levels (RPKM) of replicates within each condition ordered in the same way as $\log_2$ fold change **(A)**. Genes that are not significant (adjusted p-value above 0.01) in any experimental condition were filtered out. **(C-C')** RPKM **(C)** and $\log_2$ fold change **(C')** for juvenile hormone metabolism-related genes. Adjusted p-value was directly performed via DESeq2. *,

p.adjust<0.05; **, p.adjust<0.01; ***, p.adjust<0.001; ****, p.adjust<0.0001. FruM binding information is listed in the supplementary file.

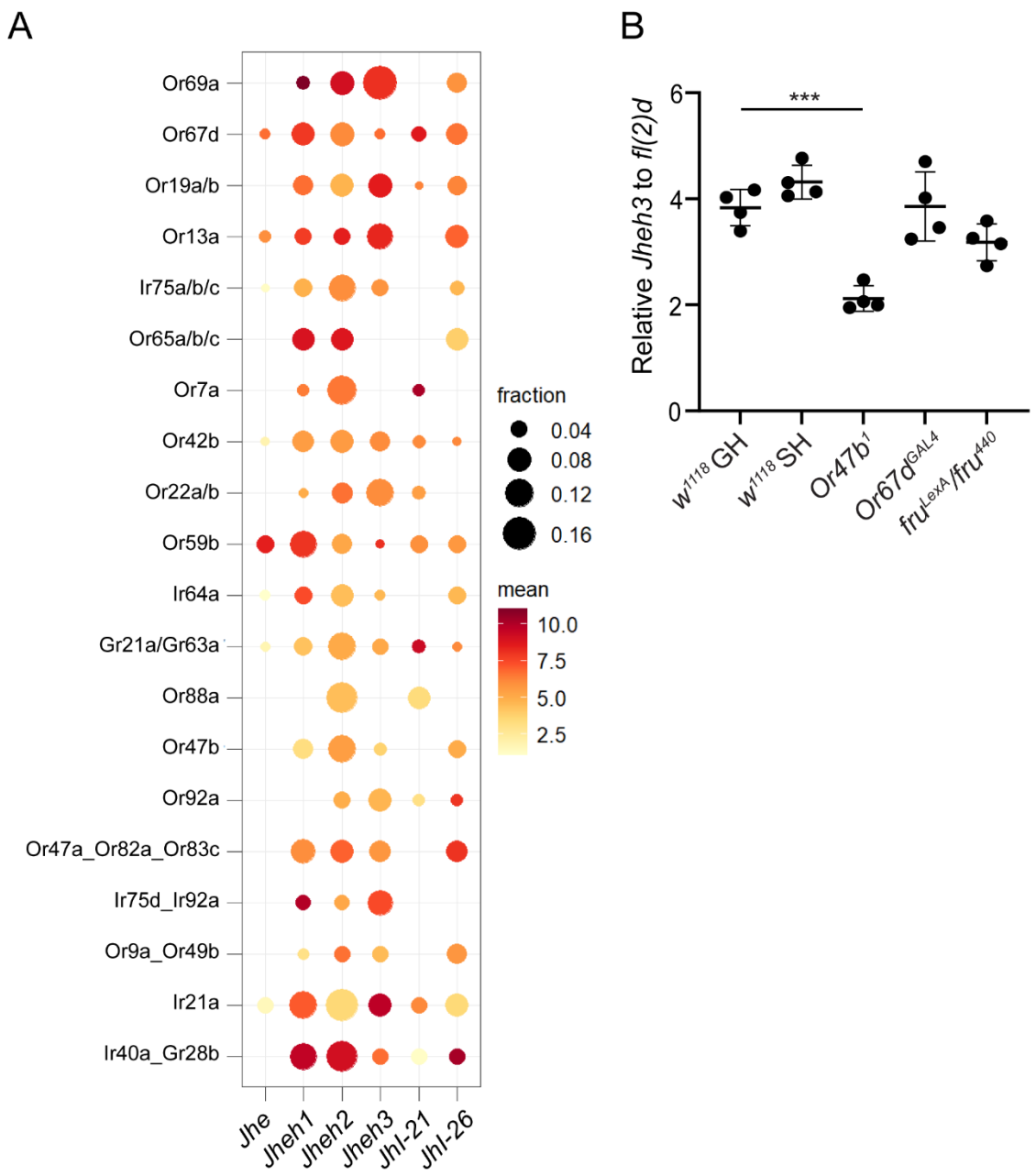

Figure 7--figure supplement 1. Validation of hormone-related gene expression across ORN classes and in mutants. (A-B) ORN class-specific expression of juvenile hormone regulators based on single-cell RNA-seq datasets from the adult ORNs (McLaughlin et al., 2021)**.** The size of each circle indicates the fraction of positive cells ($\log_2$(CPM+1)>0.5) and color intensity indicates the mean expression ($\log_2$(CPM+1)) of all positive cells. **(C)** Quantitative RT-PCR validation of *Jheh3* expression. Relative expression of *Jheh3* from antennae of

grouped and socially isolated wild types, *Or47b* mutants, *Or67d* mutants, and *fru*^{M} mutants normalized to *fl(2)d*. For each genotype or condition, n=4 biological replicates. One-way ANOVA followed by multiple comparisons (compare other groups to group-housed wild types *w*^{1118} *GH*). ***, p<0.001.

Discussion

Sensory experience influences many behaviors by modifying neural circuit function (Curley et al., 2011; Cushing and Kramer, 2005; Dey et al., 2015; Sethi et al., 2019). Yet, sensory experience-dependent molecular mechanisms that adjust circuit function and behaviors to the environment remain largely unknown. Here, we took advantage of the well-characterized and defined system of sex-specific behaviors, governed by the Fru^M, which acts as a gene regulatory switch for male-specific circuit development, function, and behavior in *Drosophila melanogaster* (Dickson, 2008; Yamamoto, 2007; Yamamoto and Koganezawa, 2013; Yamamoto and Kohatsu, 2017). Our results show that social experience and pheromone signaling alters Fru^M splicing patterns and neuromodulatory gene expression programs, which ultimately modulate circuit function and behavioral responses.

Previous studies demonstrated that social experience can modulate Fru^M-dependent sex-specific behaviors such as courtship and aggression (Curley et al., 2011; Dey et al., 2015; Sethi et al., 2019). For example, social isolation decreases the sensitivity of Or47b neurons to their pheromone ligands in a Fru^M-dependent manner, which leads to a decrease in male competitive courtship advantage (Sethi et al., 2019). Other studies have also shown that monosexual group housing can decrease the manifestation aspects of courtship behaviors such as courtship song and circling (Dankert et al., 2009). In addition to courtship, aggression behaviors which are under the control of Or67d and Or65a neurons and Fru^M function, also change with social experience (Dankert et al., 2009; Liu et al., 2011). For example, social isolation significantly increases male-male aggression (Dankert et al., 2009; Wang et al., 2008). These reports highlight the importance of social experience and pheromone signaling in the execution of sex-specific behaviors.

What are the molecular mechanisms by which Fru^M function is altered by social experience? We previously reported that social experience and Or47b signaling alters chromatin states around

fru P1 promoter (Zhao et al., 2020). These chromatin changes around *fru* with pheromone signaling and social experience modifies the levels of fru^M transcripts (Hueston et al., 2016; Sethi et al., 2019; Zhao et al., 2020). Nonetheless, the consequential details of chromatin-based changes on *fru* transcription remained unknown. One of our most intriguing findings from this transcriptome analysis is that these chromatin effects are associated with changes in the relative exon usage and splicing patterns of *fru* gene in response to impaired pheromone detection (Figure 4). Transcriptional regulation of *fru* is complex yielding 15 annotated alternatively spliced isoforms from 7 promoters giving rise to different 3' sequences which encode Zinc-finger DNA-binding domains of Fru protein (Lee et al., 2000; Meier et al., 2013; Nojima et al., 2014; Von Philipsborn et al., 2014). Alternative splicing generates Fru proteins with slightly different DNA-binding properties upstream of target genes which can have binding sites for single or multiple Fru^M isoforms (Dalton et al., 2013; Neville et al., 2014; Vernes, 2014). Many of these genes encode regulators of neural development and function. Therefore, changes in *fru* splicing patterns can affect the expression of thousands of genes simultaneously, strongly modulating neuronal responses and circuit outputs in a short period of time. Remarkably, Fru^M is expressed in ~2000 interconnected neurons highlighting a circuit for courtship behaviors from sensation to action with expression (Sato and Yamamoto, 2020; Yamamoto and Koganezawa, 2013). This expression pattern allows such neural activity-dependent influences on *fru* chromatin and transcription to propagate throughout the whole circuit. In summary, these features make circuit switch gene fru^M an efficient molecular hub onto which many internal and external factors may act to modulate circuit activity and behavioral outputs by tweaking the levels of transcripts and splicing isoforms, leading to cascade changes in transcriptional programs.

Each pheromone sensing circuit relays different information about the social environment, which is integrated and processed to output a specific behavior. Likely due to differences in neuronal identity and function, different pheromone receptors have different effects on *fru* chromatin and

splicing isoforms (Zhao et al., 2020) (Figure 4). Such sensory stimuli-dependent changes in Fru proteins can alter the expression of downstream neuromodulatory genes to have rapid, temporary, or lasting effects on neuronal activity and behavioral outputs. These changes are essential for organisms to form short/long-term adaptation to the environment. However, how these different cell types generate these differences in behavioral repertoire via changes in gene expression in the periphery have been largely unknown.

Many of the genes that show differential expression in response to social isolation and disruption of pheromone receptor or Fru^M function encode neuromodulators that affect membrane potential, such as ion channels, transporters, and neurotransmitter receptors (Figure 3; Figure 5; Figure 6). Among all conditions, social isolation possesses the fewest differentially expressed genes compared to group-housed controls with a small overlap with pheromone receptor and *fru^M* mutants. This might be due to differences in gene expression changes in response to disruption of spontaneous activity of pheromone sensing olfactory neurons with socially isolation versus disruption of both spontaneous and evoked activity in pheromone receptor mutants. Loss of Fru^M alters the expression of many neuromodulatory genes with known Fru^M binding sites in a bimodal way, suggesting Fru^M can act as both an activator and repressor of gene expression. Some of these differentially expressed genes are also altered in pheromone receptor mutants, generally in the same direction (Figure 2D,E). There are also unique overlaps between *Or47b* and *fru^M* mutants, between *Or67d* and *fru^M* mutants, and between *Or47b* and *Or67d* mutants (Figure 2B,E). Many of these differentially expressed genes are known to harbor binding sites for different Fru^M isoforms. These suggest that some of the differentially expressed genes in *Or47b* and *Or67d* mutant are due to Fru^M-dependent changes, whereas others might be Fru^M-independent, caused by OR signaling and/or ORN activity.

One functionally relevant gene among the genes that show differential regulation in pheromone receptor and *fru^M* mutants is the Fru^M target gene *ppk25*, which previously was shown to modulate ORN responses in Or47b and Or67d neurons (Ng et al., 2019; Zhang et al.,

2020). *ppk25* belongs to a family of sodium channels that serve a variety of functions, from neuromodulation to detection of sensory cues. PPK protein complexes, generally are composed of multiple subunits encoded by different *ppk* genes. Many *ppk* genes contain binding sites for Fru^M isoforms in their promoter regions (Dalton et al., 2013; Neville et al., 2014; Vernes, 2014). In addition, a recent study implicated isoform-specific Fru^M-dependent regulation of *ppk25* and *ppk23* in the modulation of Or47b and Or67d responses (Ng et al., 2019; Zhang et al., 2020). According to the genetic analysis in this study, Fru^{MB} and Fru^{MC} positively regulate the expression of *ppk25* and *ppk23*, respectively. There are apparent discrepancies with this interpretation and transcriptome data from our study, as well as others (Li et al., 2020; McLaughlin et al., 2021). While our transcriptome analysis agrees with a regulatory role for Fru^M in *ppk25* gene regulation, the regulatory mode is repressive; that is, *ppk25* expression is upregulated in *Or47b*, *Or67d,* and *fru* mutants. This type of repressive role for Fru^M in transcription also is in consensus with previous studies demonstrating Fru^M interactions with transcriptionally repressive histone-modifying enzymes such as HDAC1 (Ito et al., 2013, 2012). In addition, we are not able to detect any transcripts for *ppk23* in the antennae, and the expression of *ppk23* does not change in *Or47b*, *Or67d,* and fru^M mutants. Instead, we noticed other *ppk* genes such as *ppk6*,*7*,*13*,*14*,*15*,*19* are altered in different mutant conditions. Fru^M seems to have a bidirectional role in regulating *ppk* gene expression, where it activates the expression of a subset of *ppk* genes (*ppk7*,*13*,*14*,*15*) while repressing the expression of others (*ppk6* and *ppk25*). One way to reconcile these differences is that multiprotein PPK complexes composed of combinations of different PPK subunits, and the stoichiometric levels of each *ppk* transcript in a given neuron can determine channel function. For example, misexpression of *ppk23*, which normally is not expressed in the antennal ORNs, can interfere with PPK channel function by disrupting the existing functional complexes in a given neuron or forming new PPK complexes, thus affecting physiological properties. Another possibility is that the slight upregulation of *ppk25* in *Or47b* and fru^M mutants as well as large changes in *Or67d* mutants

may be due to global *fru*^{M} changes in the whole antennae, or through retrograde neuromodulatory signaling from the antennal lobe.

Antennal sensilla contain cell types other than ORNs, such as glia-like cells and support cells of sensillum, as well as epithelial cells. Since our transcription data is from the whole antennae, one possibility we cannot exclude is that differences in antennal gene expression in different genetic and social conditions are readouts from non-neuronal cells. Even though we anticipate the immediate effects of *Or67d* and *Or47b* mutants to happen in the ORNs expressing these two receptors, signals from ORNs can lead to secondary changes in gene expression in non-neuronal cells within the sensillum. This also brings to light a general issue with bulk tissue and whole gene knockouts where large cell-type-specific changes may be masked by cell-nonautonomous changes in gene expression from others cell types, as well as retrograde feedback signaling within olfactory circuits. Regardless, our data shows many of the differentially expressed genes encode regulators of neuronal function and neuromodulation. This increases the likelihood that the transcriptional changes in response to social and pheromonal cues are happening mostly in the neurons that respond to social cues, such as Or47b and Or67d ORNs. Future single-cell chromatin and transcription profiles from Fru^{M}-positive neurons in the antenna and brain will provide deeper insights to neuron-specific changes in gene regulation from the peripheral to the central nervous system that modulate circuit function in response to social cues.

Material and Methods

Fly genetics and genotypes

Flies were raised on standard fly food (containing yeast, cornmeal, agar, and molasses) at 25°C in a 12-hour light/12-hour dark cycle in cylindrical plastic vials (diameter, 24 mm and height, 94 mm). For social isolation (single housing, SH) condition, 80-100 hour-old pupae were separated by sex and males were placed into individual vials, allowed to eclose alone, and aged to 7 days to deprive flies of pheromone interaction on ORNs. For group housing (GH) condition, 25-30 newly eclosed males were collected and placed into food vials. These were aged to 7 days and 180 antennae were dissected per sample, for a total of 3 samples for w^{1118} *GH*, 3 samples for w^{1118} *SH*, 3 samples for $Or47b^{1}$ mutants ($Or47b^{1}/Or47b^{1}$; *Or47b-GAL4, UAS-mCD8GFP/ Or47b-GAL4, UAS-mCD8GFP*), 3 samples for $Or67d^{GAL4}$ mutants (*UAS-mCD8GFP/ UAS-mCD8GFP;* $Or67d^{GAL4}/Or67d^{GAL4}$), and 2 samples for fru^{LexA}/ fru^{4-40} mutants (w^{+}; +/+; fru^{LexA}/ fru^{4-40}).

RNA-seq

RNA-seq was performed as described before (Li et al., 2016). Male flies are aged for 7 days and dissected for the third antennal segment (~180 antennae per genotype). RNA was extracted from dissected tissues samples using Qiagen RNA-easy extraction kit, quantified using a Qubit RNA assay kit and checked for quality using a High Sensitivity RNA ScreenTape on a TapesStation (Agilent). RNA integrity scores are typically 7.0 and greater. 1ug of RNA was used to construct libraries for sequencing using a KAPA mRNA library prep kit with polyA RNA selection. Barcoded libraries are sequenced on a Novaseq 6000 SP 50 bp following manufacturer's instructions (Illumina). After demultiplexing sequence quality was assessed using FASTQC (Version 0.11.9). While there are issues with under-clustering of the samples and unbalanced pools, the data quality was typical for RNA extracted from fresh frozen material. The unbalanced pools resulted in differences in sequencing depth of each sample.

Analysis of RNA-seq data

Once sequenced, the reads are preprocessed with FASTP (S. Chen et al. 2018) to remove adaptors and trim/filter for quality. These are mapped to the dm6 reference genome using MapSplice2 (Wang et al. 2010), with individual mapping rates exceeding 98% in all cases. This raw alignment was deduplicated and filtered for mapping quality and correct pairing; additional alignments are generated to confirm results are robust to mapping ambiguity. Mapped reads are assigned to genes in the annotation using the feature Counts command from the SubRead package (Liao, Smyth, and Shi 2014). Differential expression was modeled using DESeq2 (Love, Huber, and Anders 2014) using the “apeglm” shrinkage estimator, and data was processed and visualized in R using the tidyverse framework (Wickham et al 2019), supplemented with the biomaRt (Durinck et al 2009), ComplexHeatmap (Gu, Eils, & Schlesner, 2016) and UpSet (N. Gehlenborg 2019) packages. The bioinformatics pipeline was implemented in Snakemake (Köster, J., & Rahmann, S. 2012). Code for the analysis is deposited on GitHub (https://github.com/csoeder/VolkanLab_BehaviorGenetics/tree/master/scripts). DEXSeq was used to test for differential exon use under models corresponding to those used in differential gene expression (Anders et al., 2012). From the genome-wide test, the *fruitless* locus was examined in particular. Further post-hoc t-tests are processed on non-normalized data of individual read count comparisons to look for differences at individual exons.

Statistical analysis

Adjusted p-value were directly calculated from DESeq2 or DEXSeq. Other statistical analysis is described in the legend of corresponding figures.

Specifically, to compare the exon usage in Figure 4, we also calculated p-value from t-tests of independent comparisons of group-housed male antennae transcriptome to each experimental condition at an individual exon segment (1-22, see the table below). Even though many exons level differences were significant using this method, adjusted p-value from DEXSeq gave rise to fewer significantly altered exon levels.

t-test p-value	*w*^{1118} *GH* vs			
Exon	*w*^{1118} *SH*	*Or47b*^{1}	*Or67d*^{GAL4}	*fru*^{LexA}/*fru*$^{4-40}$
P1 (1)	0.5696	0.0316	0.0619	0.0764
Male (2)	0.6843	0.0292	0.0125	0.0013
Female (3)	0.0697	0.3486	0.6932	0.5993
P2 (4)	Expression too low to calculate			
P6 (5)	Expression too low to calculate			
P3 (6)	Expression too low to calculate			
Exon 7 (7)	Expression too low to calculate			
Exon 8 (8)	Expression too low to calculate			
PD (9)	Expression too low to calculate			
P4 (10)	0.8788	0.0387	0.3002	0.1493
P5 (11)	Expression too low to calculate			
C1 (12)	0.5489	0.0117	0.1050	0.0498
C2 (13)	0.6455	0.0826	0.0007	0.0079
C3 (14)	0.3622	0.1247	0.0399	0.0650
C4 (15)	0.8295	0.3811	0.0704	0.1205
D (16)	0.7090	0.2796	0.2787	0.7042
C5 (17)	0.7575	0.7338	0.2378	0.3174
3’UTR (18)	0.7235	0.0086	0.0754	0.1328
FruA (19)	0.5241	0.0498	0.0171	0.0878
FruB (20)	0.8142	0.4809	0.7873	0.2550
FruMC male (21)	0.8519	0.1819	0.1969	0.1943
FruFC female (22)	0.4168	0.1123	0.8214	0.0223

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Quantitative reverse transcription PCR (qRT-PCR)

The qRT-PCR protocol was modified based on the previous protocol of the Volkan lab (Li et al., 2016). For each genotype, four biological replicates were prepared separately, with each replicate containing 100 antennae from 50 males (7d old). Total antennae RNA was extracted using the RNeasy Mini Kit (QIAGEN, 74104) and treated with DNase I (TURBO DNA-free Kit, Invitrogen, Thermo Fisher Scientific AM1907) to remove genome DNA. cDNA was generated from the reverse transcription of 80-150ng total RNA using the SuperScript IV First-Strand Synthesis Kit (Invitrogen, 18091050) and poly d(T) as transcription primers. qPCR was performed using the FastStart Essential DNA Green Master kit (Roche, 06924204001) on LightCycler® 96 instrument (Roche, 05815916001). The primers are listed in the table below. The expression level was calculated by ∆Ct method using the fl(2)d as the standard gene. The calculation was performed in GraphPad Prism software.

Primers Sets used in qRT-PCR assays:

Primer Names (F: Forward; R: Reverse)	Sequences
fl(2)d set15 F (exon spanning)	AGAAATCGCAGTCGGAGTT
fl(2)d set15 R	CCTTCTCAAGCGTTTGTATGC
wkd set34 F	AATGTGCTAAAGGCCTACTC
wkd set34 R (exon spanning)	TGCAGGTATACATCGCACA
ppk25 set11 F (exon spanning)	CTGCAGTATTACAGTCCCTACC
ppk25 set11 R	TCCGGATACTGTGCAGATTG
5-HT2A set15 F (exon spanning)	CCGTTCTTGGTCTGGTCAAT
5-HT2A set15 R	CGTCAATGCGTATGTGGTAAC
Jheh3 set10 F (exon spanning)	GACCGAAATTCAGGGCTTG
Jheh3 set10 R	GGTTAGCATGGGTATAAAGTCG
fru SM set7 F	ATGGGCACCTGCACAGC
fru SM set7 R	TGCCCGCACATCCGTTT
fru SF set1 F	TGAGAATTCGAGGACGTGTG
fru SF set1 R	GTTCGGGTTGAGTGTTGATTG
fru C1 set1 F	AGGAGCGATGGACCAGCAATTC
fru C1 set1 R	TTGACTGTTTCGCCCTCGCA
fru C2 set2 F	ACAGAACCAGCATCCACATCC
fru C2 set2 R	CTCGGCCGTCTTGAGAAACAT
fru C3 set2 F	GCAGCTGGATTATAGCAACAAGGA
fru C3 set2 R	CTCCCTATTGCTGCTGCTGTT
fru C4 set1 F	TGACCACAAGCGTTCATCT
fru C4 set1 R	CCATCAGCTCCTGCTTGA

fru exonD set7 F	CGTCAACCACCACAACAA
fru exonD set7 R	CTTGCCCACATTTATGGTGA
fru C5 set2_2 F	ACGCGGAGGATATGTCAA
fru C5 set2_2 R	GTCCAGCTGCAGTTGTTT
fru C5RA set2_1 F	CACACACACACACTCAAATAC
fru C5RA set2_1 R	GTTCAACGGATTCTTCACATC
fru exonA set6 F	TGGCACCATGCCAATATC
fru exonA set6 R	TCCGCATGCTTGATCTTAC
fru exonB set2 F	CACATCCGCAACCACTAC
fru exonB set2 R	GTACATGGGATGCTTGAACT
fru exonC set4 F	CGCTTGGTTATGGCAATC
fru exonC set4 R	GGTACGACATGGTGTGAT
fru exonCF set1 F	ACCAAGCAGTCAGAAACC
fru exonCF set1 R	GCCGATTACAAGTGGATGTA

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Data availability

All relevant data are within the paper and its supporting information files. Raw sequencing data will be uploaded to the GEO under embargo pending the submission and acceptance of the data submitted here for publication.

Acknowledgement

We are grateful to Liqun Luo and Hongjie Li for sharing the single-cell RNA-seq data from adult ORNs prior to publication. We would like to thank Yetong Huang, George Thomas Barlow, and Paulina Guerra-Schleske for their contributions to sample collection and RNA extraction of samples, and the Volkan lab for help with the manuscript. We thank the Bloomington Stock Center and UNC High Throughput Sequencing Facility for their services.

Funding statement

This study was supported by National Institute of Health grant number R01NS109401 and National Science Foundation award number 2006471 to PCV. Funders had no decisions in the design of the study, collection of the data or analysis, where the publication is submitted or any hand in writing the manuscript. No conflicts of interest are found.

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